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Preliminary communication

# Heparin–polynitroxides: Synthesis and preliminary evaluation as cardiovascular EPR/MR imaging probes and extracellular space-targeted antioxidants

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ABSTRACT

We report here the synthesis of heparin–polynitroxide derivatives (HPNs) in which nitroxide moieties are linked either to uronic acid or glycosamine residues of the heparin macromolecule. HPNs have low anticoagulant activity, possess superoxide scavenging properties, bind to the vascular endothelium/extra-cellular matrix and can be detected by EPR and MRI techniques. As the vascular wall-targeted redox-active paramagnetic compounds, HPNs may have both diagnostic (molecular MRI) and therapeutic (ecSOD mimics) applications.



## 1. Introduction

Cyclic nitroxides are stable free radicals having many potential biomedical applications. Specifically designed cyclic nitroxides may become unique tools for the *in vivo* functional magnetic resonance (MR) imaging (e.g. measurement of local tissue redox state) and electron paramagnetic resonance (EPR) imaging (e.g. mapping of tissue pH and oxygen concentration) [1–6]. Cyclic nitroxides, especially 2,2,6,6-tetramethylpiperidine-1-oxyl (TEMPO) derivatives, are known to scavenge superoxide anion radicals, thiyl radicals, hydrogen peroxide and myeloperoxidase (MPO)-derived oxidants [7–10]. In animal experiments, it has been shown that cyclic nitroxides effectively protect biological tissue from the reactive oxygen species (ROS)-mediated injury [11]. Major limitation of cyclic nitroxides for MR imaging is a short *in vivo* half-life time (usually, minutes) which is due to the rapid intracellular one-electron reduction into corresponding hydroxylamines. Additionally, relatively high concentrations of nitroxides are necessary to obtain a good image; these concentrations can lead to the consummation of intracellular redox equivalents and interfere with the mitochondria respiratory chain [12].

A number of physiological and pathological processes are associated with the strictly compartmentalized ROS overproduction (e.g. mitochondrion or extracellular space). Accordingly, the development of targeted antioxidants (and diagnostic probes) is a currently accepted strategy. Recently, the mitochondria-targeted antioxidants were successfully tested in a range of animal models [13,14]. In pathophysiological situation, ROS are often produced extracellularly: enzymes, such as phagocytic NADPH oxidase, xanthine oxidase (XO) and MPO selectively generate ROS on the cell surface and extracellular matrix. The ROS levels in the extracellular space are physiologically regulated by the

*Abbreviations:* HPNs, heparin–polynitroxide derivatives; EPR, electron paramagnetic resonance; MR, magnetic resonance; ecSOD, extracellular superoxide dismutase; TEMPO, 2,2,6,6-tetramethylpiperidine-1-oxyl; MPO, myeloperoxidase; ROS, reactive oxygen species.

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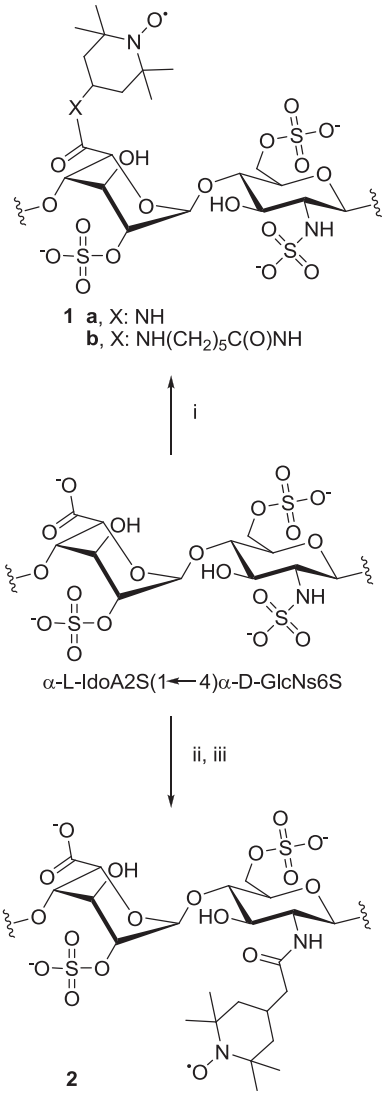


**Scheme 1.** Synthesis of compounds **1a**, **1b** and **2**. Reagents and conditions are following: (i) EDC, SuOH, 4-amino-TEMPO or 4-[(5-aminopentyl)carbonylamino]-TEMPO (**3**), $H_2O$, 0 °C → rt; (ii) Partial N-desulfation; (iii) $SuOOCCH_2$–TEMPO (**4**), DMSO–$H_2O$ (2:1), pH 8, rt.

tissue-bound extracellular Cu/Zn-superoxide dismutase (ecSOD), Cu-ceruloplasmin and different low molecular weight antioxidants. However, when ROS levels exceed the antioxidant capacity of tissue, the appearing oxidative stress can trigger the development of cardiovascular diseases, such as atherosclerosis [10,15–17]. Therefore, the extracellular space is another strategically important site to be considered both for diagnostic and therapeutic intervention. It was reported recently, that protein–nitroxide complexes (albumin–polynitroxide and hemoglobin–polynitroxide) have an increased *in vivo* half-life time and can be used for MR and EPR imaging [18,19]. However, after injection to animals these high molecular weight nitroxides remain in the circulation and lack tissue specificity.

Heparin is a mixture of highly sulfated anionic polysaccharides (av. mol. weight 12 kDa) which is composed of repeating disaccharide units of α-L-ido- or β-D-glucopyranosiduronic acids (1 → 4) linked to N-sulfo-D-glucosamine [20]. In addition to being widely used as an anticoagulant, heparin is known to have high affinity for a variety of vascular extracellular structures and has been used as a carrier for the targeted delivery of stem cells and drugs [21,22]. We hypothesized that heparin–polynitroxide (HPNs) may become a useful tool for the targeted delivery of nitroxides to the endothelial cell surface and vascular extracellular matrix. The conjugation of heparin with nitroxide can be performed in different chemical ways and may result in the alteration of properties of both heparin and nitroxide. The aim of this study was to synthesize different HPNs and provide a preliminary evaluation of their anticoagulant and antioxidant activities, as well as to test the HPN potential for vascular wall MR imaging.

## 2. Chemistry

The structures of HPNs (**1a**, **1b** and **2**) are shown in Scheme 1 (with α-L-idopyranosiduronic acid containing disaccharide, as an example). The synthesis of **1a** and **1b** included the formation of heparin amides via NHS/EDC activation of the heparin carboxylate groups followed by coupling with 4-amino-TEMPO or 4-[5-aminopentyl)carbonylamino]-TEMPO (**3**), respectively. The synthesis of **2** required the desulfation of N-sulfo-D-glucosamine with subsequent acylation of amino groups by activated ester 4-(succinimidooxycarbonylmethyl)-TEMPO (**4**). Four samples of HPNs were synthesized: $\mathbf{1a}_{18}$, $\mathbf{1a}_{72}$, $\mathbf{1b}_{45}$ and $\mathbf{2}_{65}$ where subscripts stand for the fractions (%) of nitroxide-modified disaccharides in the heparin macromolecule. Detailed synthesis and characterization of HPNs are presented in experimental protocols section and Supplementary data.

## 3. Results and discussion

### 3.1. Anticoagulant activity

All four synthesized HPNs possessed substantially lower anticoagulant activities than the non-modified heparin (Table 1). Analysis of these data allowed us to conclude that (i) anticoagulant activity of HPNs negatively correlated with the number of the heparin-associated TEMPO groups and (ii) modification of amino groups more profoundly inhibited the anticoagulant activity of heparin (two orders) than modification of carboxyl groups (one order). These findings are in general agreement with the previous works, showing that N-deacetylation and, especially, N-desulfatation of heparin, sharply decreases its anticoagulant activity [23]. To be used as tissue protectors and MRI probes, HPNs ideally, should lack the anticoagulant activity to exclude bleeding. While the anticoagulant properties of HPNs require further detailed studies, our present data suggest that **2** is more suitable for this purpose than **1**. On the other hand, we speculate that a modest degree of heparin modification by TEMPOL (similar to $\mathbf{1a}_{18}$), might be

**Table 1**
Comparison of anticoagulant activity of different HPNs. Activated Partial Thromboplastin Time (APTT) and Anticoagulation factor Xa (Anti-Xa) were measured after addition of HPNs to human blood plasma.

| Sample | Concentration (mg/L) | APTT (s) | Anti-Xa (IU/ml) |
|---|---|---|---|
| Plasma | – | 31.4 | 0.09 |
| +Heparin | 1 | 49.2 | 0.49 |
| +$\mathbf{1a}_{18}$ | 1 | 37.3 | 0.17 |
| | 12.7 | >120 | 1.15 |
| +$\mathbf{1a}_{72}$ | 1 | 31.7 | 0.08 |
| | 14.4 | 72.5 | 0.13 |
| | 144 | >120 | 0.74 |
| +$\mathbf{1b}_{45}$ | 1 | 33.2 | 0.08 |
| | 13.6 | 99.6 | 0.28 |
| +$\mathbf{2}_{65}$ | 1 | 31.0 | 0.07 |
| | 14.2 | 42.1 | 0.10 |
| | 142 | 99.5 | 0.15 |